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TRYPSIN INHIBITION OF PHOTOSYSTEM II

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SUMMARY

1. Brief incubation of chloroplasts with trypsin accelerated (by uncoupling) while longer incubation inhibited the Hill reaction with 2,6-dichlorophenolindophenol (DCIP). When an uncoupler (NH_4Cl or methylamine·HCl) was present, or when the reaction was light-limited, even brief incubation with trypsin inhibited the reaction.

2. Electron transport from ascorbate and DCIP to methyl viologen, in the presence of 3,4-dichlorophenyl-1,1-dimethylurea (DCMU), was unaffected by trypsin treatment.

3. Addition of 0.35 M Tris–HCl to normal chloroplasts severely inhibited the reduction of DCIP: after a brief transient reduction of DCIP in high light, the inhibition was virtually complete. DCIP reduction was partially restored (approx. 50 %) by the donor, 1,5-diphenylcarbohydrazide. Trypsin treatment abolished both the transient observed in the presence of 0.35 M Tris–HCl, and the partial restoration by 1,5-diphenylcarbohydrazide.

4. Inhibition of DCIP reduction by trypsin occurred two to three times faster with water as donor as with 1,5-diphenylcarbohydrazide. In both cases, the inhibition developed with first order kinetics.

5. Both treatment with 0.35 M Tris–HCl and with trypsin lowered the relative fluorescence yield of otherwise untreated chloroplasts to the same level. After the Tris treatment alone, but not after both trypsin and Tris treatments, 1,5-diphenylcarbohydrazide addition raised the fluorescence yield. Nor could the yield of trypsin-treated chloroplasts be raised by DCMU.

6. It is concluded that trypsin(i) uncouples, (ii) does not inhibit Photosystem I between plastocyanin and ferredoxin, and (iii) inhibits at least two sites of electron transport on the oxygen producing side of Photosystem II. A more rapid inhibition occurs between the sites of water oxidation and 1,5-diphenylcarbohydrazide oxidation, and a slower inhibition occurs between the sites of 1,5-diphenylcarbohydrazide oxidation and Photosystem II.

INTRODUCTION

MANTAI[1] reported that trypsin digestion of whole chloroplasts affects electron transport in two ways. After brief treatments, the rate of electron transport to 2,6-

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCMU, 3,4-dichlorophenyl-1,1-dimethylurea.

dichlorophenolindophenol (DCIP) increased, presumably as a result of uncoupling. Longer treatment with trypsin revealed an inhibition of electron transport. Our study was undertaken in order to identify and locate sites of trypsin inactivation more exactly. For this, we have examined the effects of trypsin on both electron transport and chlorophyll fluorescence — especially in System I and System II partial reactions with donors. Recently, MANTAI[2] reported no trypsin inhibition of $NADP^+$ reduction by ascorbate–DCIP donor system. Our principle results for other System I reactions also show no inhibition by trypsin but some effects of trypsin on $NADP^+$ reduction have been found by us in preliminary experiments. In System II, we have obtained evidence of two inhibitions both on the oxidizing side.

METHODS

Chloroplast preparation

Chloroplasts were routinely isolated from two to three week old oat seedlings (*Avena sativa*, variety Gary) or market spinach (*Spinacia oleracea*) at 0–4°. Approx. 50 g of leaves were ground in a Waring blender at full speed for 15 sec in 150 ml of buffer containing 0.01 M Tris–HCl (pH 7.6), 0.4 M sucrose, and 0.01 M NaCl. The supernatant was filtered through 10 layers of cheese cloth, a glass wool plug, and centrifuged for 10 min at 600 × *g*. The pellet was resuspended in 40 ml of the above buffer and centrifuged at 300 × *g* for 1 min. The pellet was dicarded and the supernatant centrifuged again for 10 min at 2000 × *g* to sediment chloroplasts. This pellet was resuspended in 1–3 ml of the above buffer (2–3 mg chlorophyll per ml) and stored in ice. Chlorophyll concentration was determined by the method of ARNON[3].

Trypsin treatment

Trypsin treatment was performed as follows. Two 45-ml volumes containing chloroplasts, 30 μg chlorophyll per ml, 0.01 M Tris–HCl (pH 7.6) and 0.01 M NaCl were pre-incubated at 25° for 3 min, with magnetic stirring. At time zero, trypsin (grade XI, obtained from Sigma Chemical) was added (0.13 μg trypsin per μg chlorophyll) to one suspension (denoted as "trypsin treated") while to the other an equivalent volume of buffer was added (denoted as "control"). After varying periods of incubation, 5.0-ml quantities were withdrawn and transferred to test tubes containing 0.02 ml of trypsin inhibitor (2 mg/ml) (Grade 1-s, obtained from Sigma Chemical) and cooled to 0°. Both the control and trypsin treated chloroplasts received the same volume of trypsin inhibitor. These chloroplasts were assayed for activity.

DCIP and $NADP^+$ reduction

DCIP reduction was measured in a cross-beam spectrophotometer similar to that described by LIEN[4]. The weak measuring beam passed in sequence through a Baird Atomic 583-nm interference filter, a Corning glass filter No. 3387, a Bausch and Lomb 0.3 *A* neutral density filter, the sample cuvette (1 cm), Corning filters No. 9788 and No. 3482 (to reduce scattering of the actinic beam), and finally, was transmitted by a Bausch and Lomb monochrometer (set at 590 nm) to an RCA IP21 photomultiplier. Perpendicular to the weak measuring beam, a strong actinic beam was obtained from a 1000-W tungsten projection lamp. The broad band actinic beam was carefully freed of infrared and passed through either two Corning filters No. 3484 or one

Kodak gelatin filter No. 77A to the sample cuvette. The latter was maintained at 18°. A calibrated thermopile behind the cuvette gave illuminating intensity. The extinction coefficient for DCIP at 590 nm was assumed to be 19.8 $mM^{-1} \cdot cm^{-1}$ (ref. 4).

$NADP^+$ reduction was measured in the same instrument. The weak measuring beam in this case passed through a Corning filter No. 5850 before the sample cuvette and the monochrometer was set at 340 nm. No filters were needed between the cuvette and the monochrometer to reduce scattering effects. The extinction coefficient for $NADP^+$ at 340 nm was assumed to be 6.22 $mM^{-1} \cdot cm^{-1}$ (ref. 5).

The "log converter" circuits of LIEN[4] were employed so that absorbance of the sample could be displayed directly on a 10" recorder.

O_2 consumption

Methyl viologen-catalyzed oxygen consumption was measured polarographically with a Clark-type electrode (YSI Model 5331).

Fluorescence measurements

Fluorescence emission was measured in the same apparatus used for measuring DCIP reduction. A weak broad-band blue exciting beam was isolated with two Corning filters No. 4303, one No. 4308, and an 0.6 *A* neutral density filter. Fluorescence was measured at right angles to the exciting beam after passing the emitted light through a Baird Atomic 680 nm interference filter and the monochrometer (also 680 nm). The signal was amplified and recorded on a strip chart recorder.

RESULTS

Effect of trypsin on DCIP reduction

In experiments with coupled chloroplasts, the rate of DCIP reduction was a somewhat variable function of the duration of trypsin treatment. Fig. 1 (bottom two

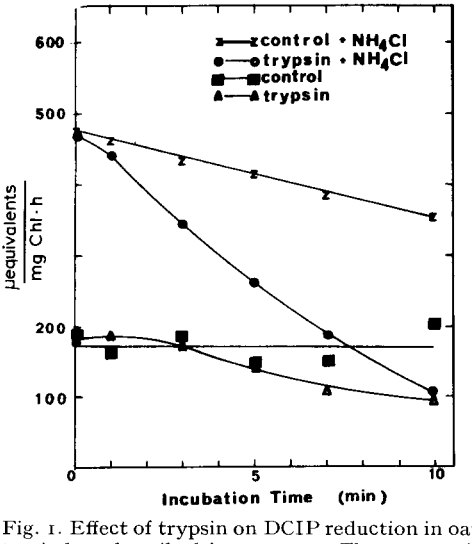

Fig. 1. Effect of trypsin on DCIP reduction in oat chloroplasts. Oat chloroplasts were prepared and treated as described in METHODS. The assay mixture contained NaCl, 0.01 M; Tris–HCl, 0.01 M (pH 7.6); DCIP, 40 μM; chloroplasts at 10 μg chlorophyll per ml and (where indicated) NH_4Cl, 2 mM, in a total volume of 3.0 ml. Incident illumination of about $5 \cdot 10^5$ $ergs \cdot cm^{-2} \cdot sec^{-1}$.

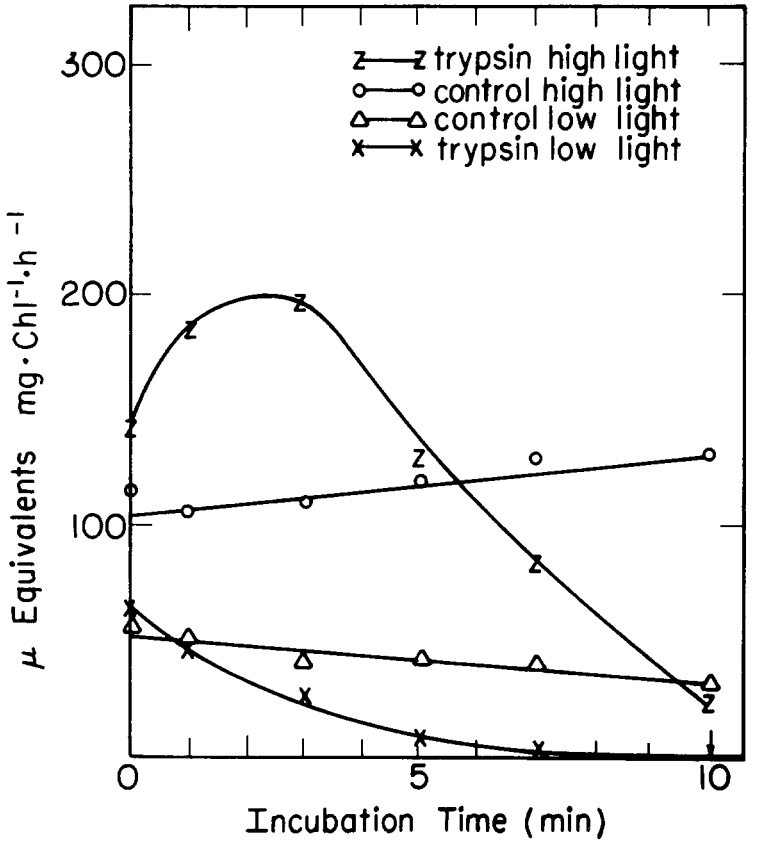

Fig. 2. Effect of trypsin on DCIP reduction in oat chloroplasts. Conditions as in Fig. 1. Low illumination was about $3 \cdot 10^4$ ergs·cm^{-2}·sec.$^{-1}$

curves) and Fig. 2 (top two curves) show two extreme variations which were observed for oat chloroplasts. In Fig. 1, the rate of DCIP reduction remained constant for the first few minutes of incubation. With progressively longer incubation times, increasing inhibition appeared. We also observed this effect in the methyl viologen Mehler reaction. In contrast, Fig. 2 shows marked stimulation of electron transport (presumably uncoupling) at early incubation times, while inhibition occurred later. This latter case was described by MANTAI[1] for spinach chloroplasts. The cause of the variations in our experiments is not known; the variations could reflect differences in the degree of coupling in different batches of chloroplasts, or, possibly, differences in relative rates of uncoupling and inhibition by trypsin.

When electron transport was not limited by a phosphorylation coupled step, the inhibition of DCIP reduction by trypsin began immediately. This was the case with chloroplasts in saturating light in the presence of an uncoupler (NH_4Cl or methylamine–HCl) (Fig. 1, upper two curves), and it was also the case in low illumination without uncoupler (Fig. 2, lower two curves). Similar results were obtained in the reactions with methyl viologen, $NADP^+$, and $Fe(CN)_6^{3-}$. In all cases, trypsin inhibition appeared to be a first order kinetic process.

Effect of trypsin on System I electron transport

Fig. 3 shows the absence of an effect of trypsin on the Photosystem I reaction consisting of electron transport from ascorbate–DCIP to methylviologen in the presence of 3,4-dichlorophenyl-1,1-dimethylurea (DCMU). In this case, trypsin treatment did not inhibit, and this was true for both high and low concentrations of the donor system. With the same chloroplasts, trypsin treatment produced a typical, strong inhibition of electron transport from water to DCIP in the presence of uncoupler.

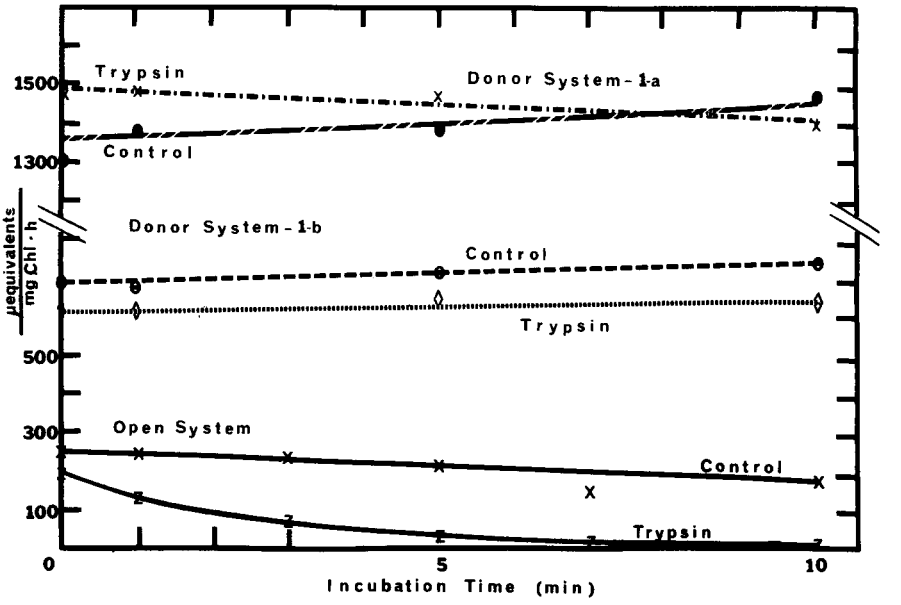

Fig. 3. Effect of trypsin on methyl viologen and DCIP reduction. Reaction mixture for the donor system assays included oat chloroplasts at 10 μg chlorophyll per ml; methyl viologen, 0.5 mM; KCN, 1 mM; DCMU, 50 μM; NH_4Cl, 2 mM; NaCl, 0.01 M and Tris–HCl, 0.01 M (pH 7.6), in 3.0 ml total volume. In addition, "Donor System-1-a" contained 1 mM ascorbate and 0.1 mM DCIP while "Donor System-1-b" contained 0.1 mM ascorbate and 0.04 mM DCIP. The samples were assayed for oxygen consumption with a Clark electrode and were illuminated with white light of about 10^6 ergs·cm^{-2}·sec^{-1}. The "Open System" (*i.e.* electron transport from water to DCIP) was assayed as described in Fig. 1, and in the presence of 2 mM NH_4Cl.

Effect of Tris–HCl on DCIP reduction

Washing chloroplasts with a high concentration of Tris–HCl severely inhibits electron transport on the oxygen-producing side of Photosystem II[6]. Several compounds have been reported which will donate electrons after the site of Tris–HCl inhibition and restore some fraction of the original activity[7,8]. Among these, 1,5-diphenylcarbohydrazide can apparently restore full activity under certain conditions[9].

Fig. 4 shows that the addition of Tris–HCl (pH 8.0) strongly inhibited the steady state reduction of DCIP. With 0.3–0.50 M Tris–HCl the inhibition was total, but about 50 % of the original activity was restored by the addition of 0.7 mM 1,5-diphenylcarbohydrazide. Over 90 % of the 1,5-diphenylcarbohydrazide–restored rate was eliminated by DCMU.

Although the steady state reduction of DCIP was completely inhibited by 0.35 M Tris–HCl (pH 8.0) there was a slow transient absorbance change that occurred

at 590 nm and lasted for about 20 sec in saturating light (see Fig. 5). The transient was not seen when DCIP was replaced with $Fe(CN)_6^{3-}$ or methylviologen: however, the lower sensitivity of the measurements of $Fe(CN)_6^{3-}$ and methylviologen reduction could explain the apparent absence. The transient was inhibited about 90 % by DCMU. It could not be repeated after a short dark time (approx. 2.0 min), and it was elimina-

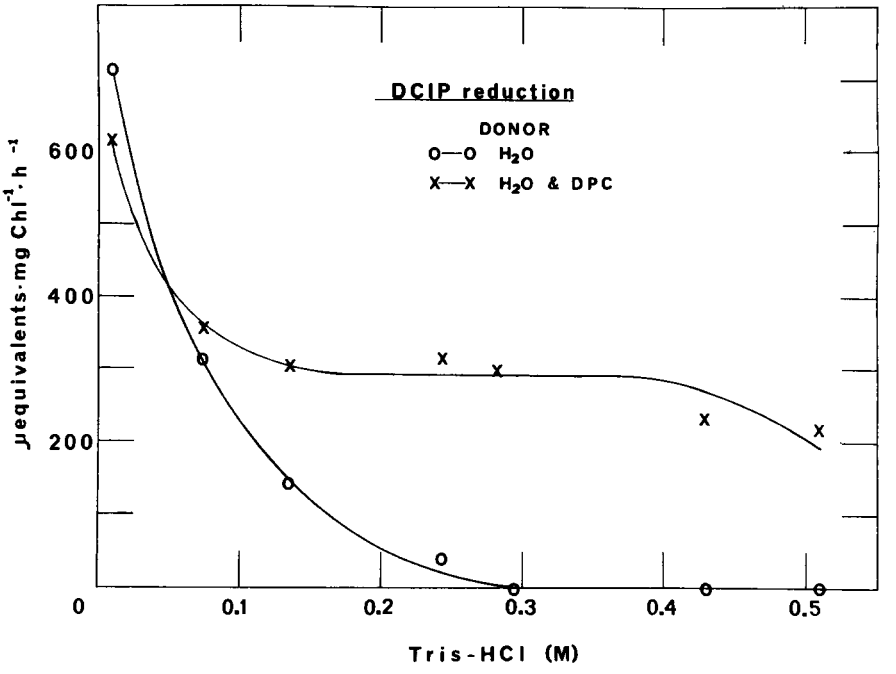

Fig. 4. Concentration curve for the effect of Tris–HCl on the reduction of DCIP. Spinach chloroplasts were prepared as described in METHODS. The reaction mixture contained chloroplasts at 10 μg chlorophyll per ml; Tris–HCl, 0.01 M (pH 7.6); NaCl, 0.01 M; methylamine–HCl, 33 mM and DCIP, 20 μM in a volume of 3.0 ml. Tris–HCl (pH 8.0) was added to desired concentrations. Incident illumination intensity was about $5 \cdot 10^5$ ergs·cm^{-2}·sec^{-1}. DPC = 1,5-diphenylcarbohydrazide.

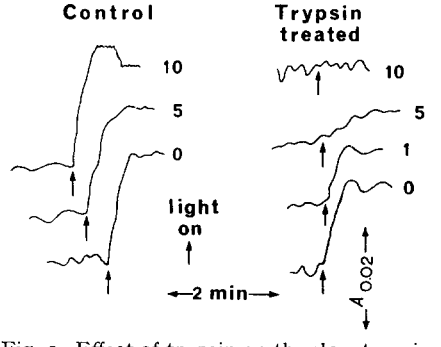

Fig. 5. Effect of trypsin on the slow transient of DCIP reduction in a high concentration of Tris–HCl. Spinach chloroplasts were treated as detailed in methods. The numbers refer to time of incubation at 25°. Samples contained chloroplasts at 10 μg chlorophyll/ml; Tris–HCl, 0.01 M (pH 7.6); NaCl, 0.01 M; methylamine–HCl, 33 mM; DCIP, 20 μM and Tris–HCl, 0.35 M (pH 8.0). Illumination intensity was about $5 \cdot 10^5$ ergs·cm^{-2}·sec.

ted when the chloroplasts were illuminated in high light preceding the addition of DCIP. Since the 590 nm absorbance change only occurs with DCIP and since the magnitude of the change is too large to be accounted for by plastocyanin reduction the change must be attributed to DCIP reduction. Fig. 5 shows typical traces observed in the presence of 0.35 M Tris–HCl. From these, and assuming the transient is due to DCIP reduction, one can calculate that the extent of the transient reaction is about 5 equiv./mole chlorophyll and that the average rate of reduction during the transient is about 1–10 the control rate in the absence of Tris–HCl. Since the transient reaction is eliminated by DCMU, the electron flow must originate from the oxygen-producing side of System II. Two possible explanations for this transient electron flow in the presence of a high concentration Tris–HCl are: (1) there may exist a small pool of reductant located on the oxygen-producing side of System II and after the site of Tris inhibition, capable of donating electrons at a slow rate, and (2) inhibition by Tris–HCl may require a light period for completion. Fig. 5 illustrates that trypsin treatment abolishes the transient reaction. Thus, the donor pool is made unavailable by trypsin action (or Tris inhibition is completed in darkness in the presence of trypsin).

Comparative kinetics of trypsin inhibition of reactions with water and 1,5-diphenylcarbohydrazide as donors

Fig. 6a shows the rates of DCIP reduction by water (with untreated chloroplasts) and by 1,5-diphenylcarbohydrazide (with chloroplasts treated with 0.35 M Tris) as functions of the duration of trypsin treatment. Fig. 6b shows that, in both cases, trypsin inhibition develops with first order kinetics, and, moreover, that the rate constant for the inhibition of the reaction with water as donor is 2–3 times greater

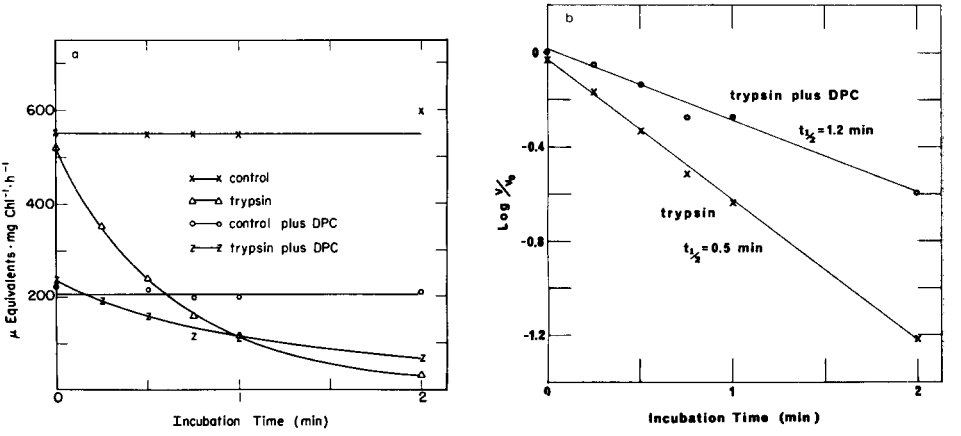

Fig. 6. Differential effect of trypsin on DCIP reduction. Spinach chloroplasts were treated as described in text with the exception that the trypsin and trypsin-inhibitor concentrations were increased five fold. Samples assayed for DCIP reduction included chloroplasts at 10 μg chlorophyll/ml, Tris–HCl, 0.01 M (pH 7.6); NaCl, 0.01 M; DCIP, 20 μM and methylamine–HCl, 33 mM. Samples assayed for DCIP reduction due to 1,5-diphenylcarbohydrazide oxidation contained in addition Tris–HCl, 0.35 M (pH 8.0) and 1,5-diphenylcarbohydrazide, 0.7 mM. Only the steady state reaction rate (not that of the transient in the first 20 sec) was measured. There was no detectable steady state reaction in the presence of 0.35 M Tris when 1,5-diphenylcarbohydrazide was omitted. The rates of the 1,5-diphenylcarbohydrazide (DPC) supported reaction are net rates after substracting the low rates of the DCMU-insensitive reaction.

than that for inhibition of the 1,5-diphenylcarbohydrazide reaction. The faster inhibition of the reaction with water was consistently observed, both in DCIP and methyl viologen reductions.

Effects of trypsin on fluorescence

Fig. 7A shows the well-known lowering of fluorescence yield in normal chloroplasts by a Hill oxidant, and the elevation of yield by subsequent addition of DCMU. (In this case with $Fe(CN)_6^{3-}$, DCMU fails to fully restore the original level because of the dye absorption of exciting fluorescent light). In Fig. 7B, the fluorescence yield is initially low after trypsin treatment and is unaffected by Hill oxidant and DCMU; these results are consistent with trypsin action on the oxidizing side of System II.

In Fig. 7C, there is a 2 min-long decline of fluorescence when normal chloroplasts in the presence of 0.35 M Tris–HCl were illuminated. The fluorescence was raised

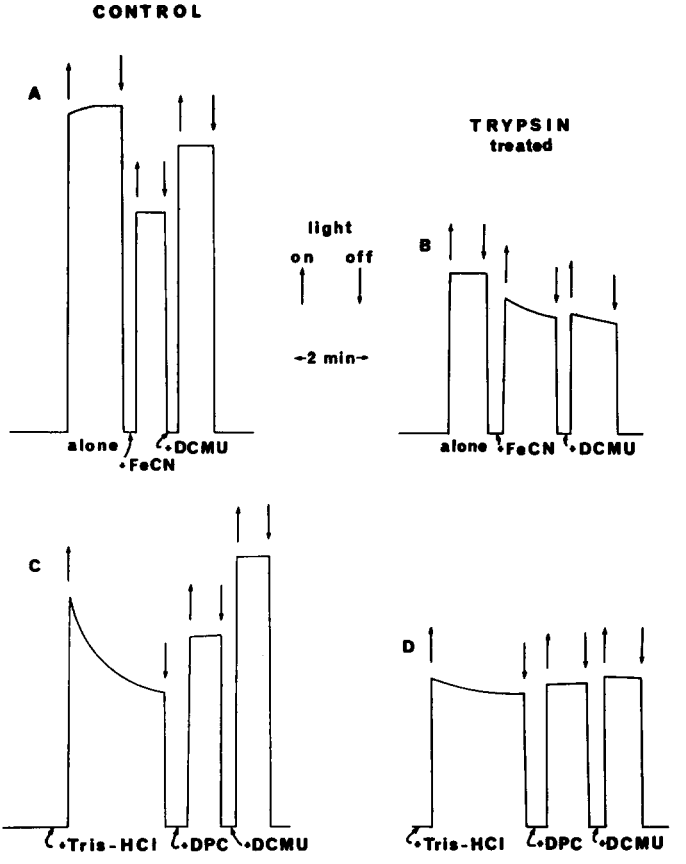

Fig. 7. Effect of trypsin on the relative fluorescence emission of spinach chloroplasts. Fluorescence emission was measured as described in METHODS. Samples assayed were the 2-min control (A and C) and the 2-min trypsin treated chloroplasts (B and D) obtained under conditions described in Fig. 6. Samples in A and B contained chloroplasts at 10 μg chlorophyll per ml; Tris–HCl, 0.01 M (pH 7.6); NaCl, 0.01 M; methylamine–HCl, 33 mM and (where indicated) $Fe(CN)_6^{3-}$, 0.2 mM and DCMU, 7 μM in a final volume of 3.0 ml. Samples in C and D contained in addition Tris–HCl, 0.35 M (pH 8.0) and (where indicated) 1,5-diphenylcarbohydrazide (DPC), 0.7 mM. Incident illumination intensity was about $1.6 \cdot 10^4$ ergs$\cdot$cm$^{-2}\cdot$sec^{-1}.

by 1,5-diphenylcarbohydrazide, and raised further by DCMU. This behavior has been previously reported by YAMASHITA AND BUTLER[6] and is taken as evidence that 1,5-diphenylcarbohydrazide donates to System II after a site blocked by Tris. Fig. 7D shows that the fluorescence of chloroplasts initially treated with trypsin is low, is not affected by the presence of Tris, and is not raised by the donor 1,5-diphenylcarbohydrazide or by DCMU. Evidently, trypsin treatment blocks electron transport between the site of 1,5-diphenylcarbohydrazide oxidation and Photosystem II.

DISCUSSION

Except in sonicated chloroplast fragments[10] or photosynthetic mutants[11], the reduction of DCIP (and presumably also methyl viologen) primarily occurs at a Photosystem I site, X, just prior to ferredoxin. System I donors (*e.g.* ascorbate–DCIP) presumably donate to plastocyanin just prior to Photosystem I. Our findings indicate that trypsin (i) has no affect on System I between plastocyanin and X, (ii) uncouples, presumably between System II and I, (iii) rapidly inhibits a step between water and the site of Tris blockage on the oxidizing side of System II, and (iv) slowly inhibits a step between the site of Tris blockage and Photosystem II, also on the oxidizing side of System II. Recently, MANTAI[2] reported no inhibition by trypsin of the System I reduction of $NADP^+$ by ascorbate–DCIP; in contrast, preliminary experiments of ours show a definite inhibition by trypsin in this reaction. Probably, trypsin attacks ferredoxin or ferredoxin–$NADP^+$ reductase. The lack of effect on electron transport from ascorbate–DCIP to methyl viologen, in the presence of DCMU, shows that the photoreactive part of System I is not attacked. As for that portion of the electron transport pathway lying between Q and plastocyanin, it is noteworthy that, except for uncoupling, trypsin apparently makes no attack, or, at least, does so slowly as to have escaped attention. As with heating, ageing, ultraviolet inactivation, and Tris, the oxidizing side of System II appears more sensitive than other portions of the electron transport pathway to trypsin inactivation.

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